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Review Article

Candidate RNA biomarkers in biofluids for early diagnosis of ovarian cancer: A systematic review

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HIGHLIGHTS

- Non-invasive, robust and reliable biomarkers for early detection of ovarian cancer are urgently needed
- Extracellular RNAs circulating in biofluids have emerged as biomarker candidates for early detection of ovarian cancer
- The transcriptome of serum, plasma, ascites and urine of ovarian cancer patients has been explored
- RNA-based signatures show potential to outperform the diagnostic performance of CA125
- Stringent validation of the reported markers is required before implementation in routine clinical care

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ABSTRACT

Ovarian cancer is often diagnosed in an advanced stage and is associated with a high mortality rate. It is assumed that early detection of ovarian cancer could improve patient outcomes. Unfortunately, effective screening methods for early diagnosis of ovarian cancer are still lacking. Extracellular RNAs circulating in human biofluids can reliably be measured and are emerging as potential biomarkers in cancer. In this systematic review, we present 75 RNA biomarkers detectable in human biofluids that have been studied for early diagnosis of ovarian cancer. The majority of these markers are microRNAs identified using RT-qPCR or microarrays in blood-based fluids. A handful of studies used RNA-sequencing and explored alternative fluids, such as urine and ascites. Candidate RNA biomarkers that were more abundant in biofluids of ovarian cancer patients compared to controls in at least two independent studies include miR-21, the miR-200 family, miR-205, miR-10a and miR-346. Amongst the markers confirmed to be lower in at least two studies are miR-122, miR-193a, miR-223, miR-126 and miR-106b. While these biomarkers show promising diagnostic potential, further validation is required before implementation in routine clinical care. Challenges related to biomarker validation and reflections on future perspectives to accelerate progress in this field are discussed.

Contents

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1. Introduction

Ovarian cancer is the fifth leading cause of cancer-related mortality in women, with five-year survival rates below 45%, largely driven by late stage diagnoses [1]. Ovarian cancer is often referred to as a 'silent killer' because local stage disease is usually asymptomatic and symptoms of advanced stage disease are nonspecific. More than 75% of affected women are diagnosed when the cancer is already in advanced stage and has spread beyond the pelvis. Early detection of ovarian cancer is key as local stage disease can be cured more effectively than late stage disease and has a 5-year survival rate of 93% [1]. Ovarian cancers are classified into various histological subtypes, with various underlying transcriptional and mutational patterns. High-grade serous carcinoma is the most prevalent and most challenging subtype to detect in genetically predisposed populations, such as germline *BRCA1/2* mutation carriers. Unfortunately, no effective screening method for this cancer entity is available. Cancer antigen 125 (CA125) testing in serum, the most studied ovarian cancer screening modality so far, has important limitations [2,3]. Less than 50% of patients with early stage ovarian cancer have elevated CA125 levels, and elevated CA125 levels can also be observed in benign conditions. It is currently impossible to diagnose ovarian cancer without surgical resection of the tumor mass. Therefore, there is an urgent clinical need for non-invasive, robust and reliable diagnostics for ovarian cancer detection.

Extracellular RNAs (exRNAs) in blood and other biofluids are emerging as potential biomarkers for a wide range of diseases [4–6]. These so-called liquid biopsies may offer a non-invasive alternative to tissue biopsies for diagnosis, prognosis and treatment response monitoring. Approaches to identify potential biomarkers for ovarian cancer screening include the characterization of circulating tumor DNA, circulating tumor cells and metabolites, amongst others. In this review we focus on the quantification of exRNAs. The repertoire of circulating RNA molecules in human biofluids is more diverse and complex than originally anticipated. A multitude of previously unknown coding and non-coding RNA species have been identified in biofluids (Fig. 1A). While messenger RNAs (mRNAs) serve as template for translation, and thus for the synthesis of proteins, the majority of the transcriptome does not code for proteins. Instead, these non-coding RNAs play regulatory roles during transcription and translation. This includes small non-coding RNAs such as microRNAs (miRNAs, 18–24 nucleotides in length) and long non-coding RNAs (lncRNAs, >200 nucleotides). More recently, circular RNAs (circRNAs) have been discovered as a novel class of non-coding RNAs with biomarker potential [7]. Human biofluids contain high levels of *endo-* and exonucleases which makes extracellular RNAs prone to degradation. As a result, degraded RNA fragments are present in fluids, which adds to the complexity of quantifying RNA. Fortunately, some RNAs in circulation are less prone to degradation because of their intrinsic stability (circRNAs), binding to carrier proteins such as Argonaute 2 (miRNAs) or encapsulation in membrane-enclosed vesicles, referred to as extracellular vesicles (EV). Accurate measurements of RNA are possible provided the methods for sample collection, processing and RNA isolation are optimized and standardized.

Three principal methods are used to measure the expression levels of exRNAs: reverse transcription quantitative PCR (RT-qPCR), microarray hybridization and RNA-sequencing (RNA-seq), each with their own strengths and limitations (Figure1B). RT-qPCR and microarrays allow RNA quantification of a predefined set of target sequences while RNA-seq is a high-throughput method that does not require any prior genomic information other than a reference genome to map the identified sequence. Because of this, RNA-seq is gaining acceptance as a routine clinical lab test [8].

Here we provide a systematic overview of RNA biomarkers detectable in human biofluids that have been studied for early diagnosis of ovarian cancer. We aim to assess agreements and disagreements of these reported RNA biomarkers and to summarize available evidence for each candidate biomarker in the context of a future implementation in routine clinical care.

2. Materials & methods

Reports of current systematic review adhere to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [9]. This systematic review was prospectively registered with the PROSPERO database (Ref: CRD42020197950). Patients were not directly involved in the conduct of this study. MEDLINE (via PubMed) and the Embase database were searched using a systematic search strategy (supplementary Table S1) from inception until 15 August 2020. Forward and backward snowballing to identify additional relevant publications was performed.

Primary literature screening was performed by two researchers (AM and EH), independently, in a blinded standardized way using the Rayyan web application [10]. Only peer-reviewed studies written in English were considered. Case-control biomarker studies focusing on RNA molecules detected in human biofluids, comparing adult patients diagnosed with ovarian cancer against control group(s) with the aim to identify diagnostic biomarkers were eligible. Only original studies that included at least 20 newly diagnosed ovarian cancer patients were included. In silico analyses entirely based on data of previous studies were excluded. Case reports, reviews, commentaries and conference reports were excluded. Studies where RNA molecules are studied (based on their presence/absence or concentration) to distinguish ovarian cancer from control group(s) using univariate or multivariate statistical models, were included.

Two independent reviewers (AM and EH) also performed the data extraction. Disagreement between reviewers was resolved by consensus. The following data were extracted from each full-text manuscript: (1) study characteristics (authors, year of publication, journal, country, publication type), (2) biofluid studied, (3) RNA biotype studied, (4) technique to quantify the RNA, (4) reproducibility of the methods (biofluid collection, RNA isolation, data analysis), (5) histological type of ovarian cancer, and the International Federation of Gynecology and Obstetrics (FIGO) classification, (6) biofluid collection prior to therapy, (7) definition of the control group, (8) number of participants in the ovarian cancer group and the control group, (8) independent validation cohort included, (9) comparison with CA125, (10) pre-defined cutoff, (11) number of differentially expressed RNAs, upregulated RNAs and down-regulated RNAs, statistical test used, *p*-value, multiple testing correction, (11) statistical model with selected RNAs, sensitivity,

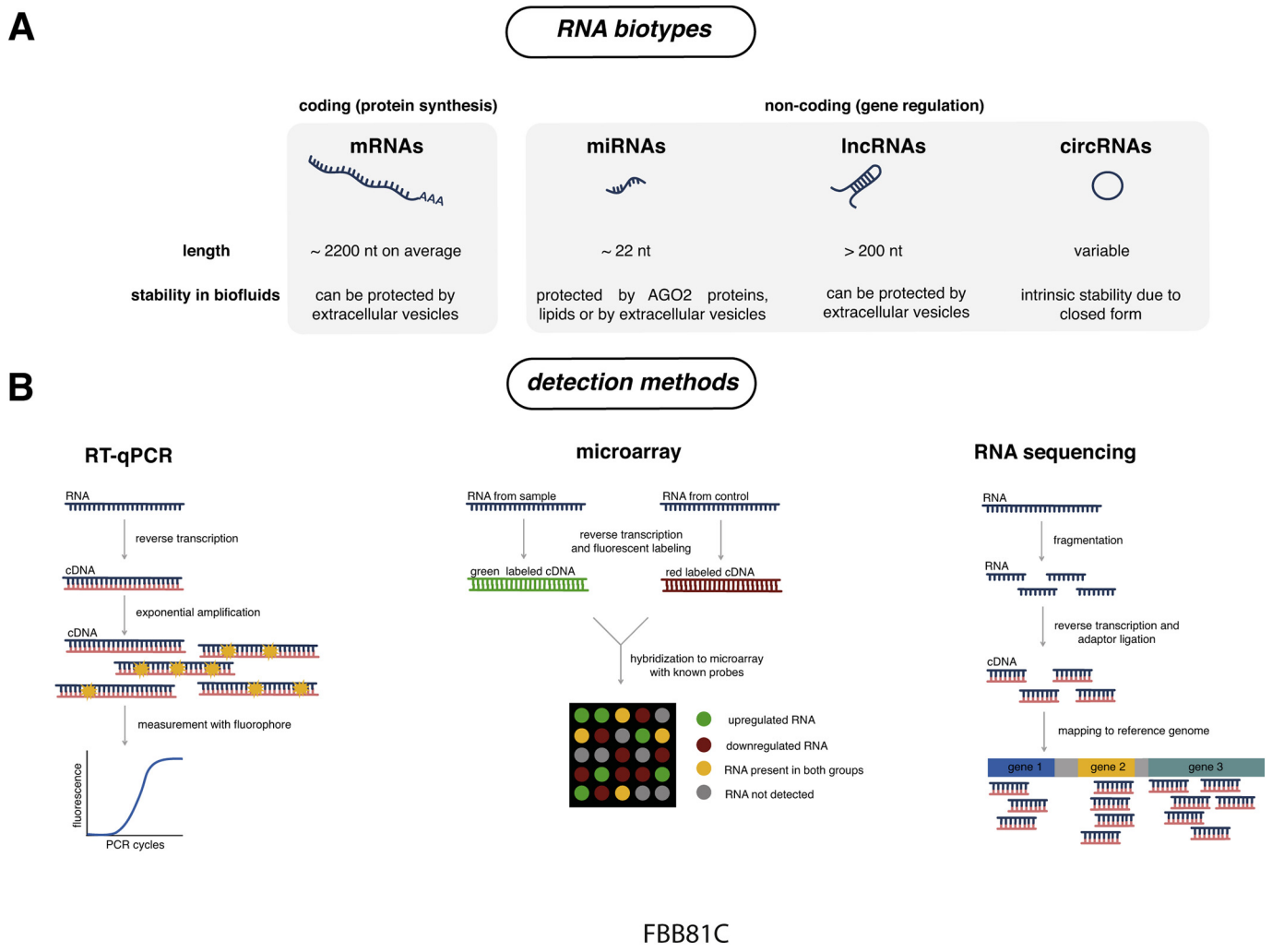

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Fig. 1. (A) Overview of different RNA biotypes identified in human biofluids. (B) Main detection methods for RNA in biofluids. Reverse transcription quantitative PCR (RT-qPCR): RNA is converted into cDNA and amplified by PCR in the presence of a target-specific oligonucleotide bound to a fluorescent probe or a fluorescent DNA binding dye. The number of PCR cycles needed to reach a detection threshold of fluorescence is inversely related to the amount of input RNA and can therefore be used to determine the relative concentration of RNA. Microarray: RNA is extracted from diseased and control samples, reverse transcribed, and fluorescently labeled (e.g. green for diseased cDNA and red for control cDNA). The cDNAs is then hybridized to a microarray chip, where they bind to complementary sequences (probes) from annotated genes. The relative amount of green versus red fluorescence corresponds to the relative expression of genes in control versus diseased samples. RNA sequencing: extracted RNA is fragmented, reverse transcribed, and extended with adapters to enable massively parallel sequencing. The resulting sequences (reads) are aligned against the reference genome to reveal the expression level of the various genes. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

specificity and area under the curve of the model. The risk of bias per study was independently assessed by two investigators (AM and EH) using the Quality Assessment of Diagnostic Accuracy Studies-2 (QUADAS-2) checklist[11].

3. Results

3.1. Study selection

The PRISMA diagram for the study selection process is shown in Fig. 2. We identified 780 records through the database search, of which 36 records met the inclusion criteria and were included for qualitative synthesis [12–47]. Characteristics of the included studies are summarized in Table 1. The detailed extraction table is provided in supplementary Table S2. All 36 records are studies with a case-control design comparing liquid biopsy samples of ovarian cancer patients and a control group. In most studies, the control group consisted of (age-matched) healthy women or women with a benign ovarian mass, benign gynecologic disease or a borderline ovarian tumor. In one study, women with solid cancers (other than ovarian cancer) were also studied as control group [13].

3.2. Risk of bias assessment

A summary of the risk of bias assessment of the 36 included studies is shown in supplementary Figure S1. All studies were judged to have a high risk of bias in at least three domains of the QUADAS-2 checklist. All 36 studies are case-control studies. Selection bias is inherent to this study design, as there are often confounding factors not specifically associated with the disease status itself, but associated with certain characteristics of the diseased patient group [48]. Initial screening of the biomarkers includes a comparison of the two groups without statistically controlling for confounding factors, potentially resulting in false discoveries. Matching is of use to adjust for known confounding factors. In 14 out of the 36 studies age-matching between cases and controls was applied. In two studies, gender nor age of the control group was reported [38,45]. Samples of ovarian cancer patients with FIGO stage I-II are most interesting to investigate in the context of early detection. All studies, except two where the FIGO classification of the patients is not mentioned, combined samples from early disease (stage I-II) and advanced disease (stage III-IV). Details on the number of samples per disease stage are provided in supplementary Table S2. Notably, biomarkers identified in patients with advanced disease are not necessarily useful in

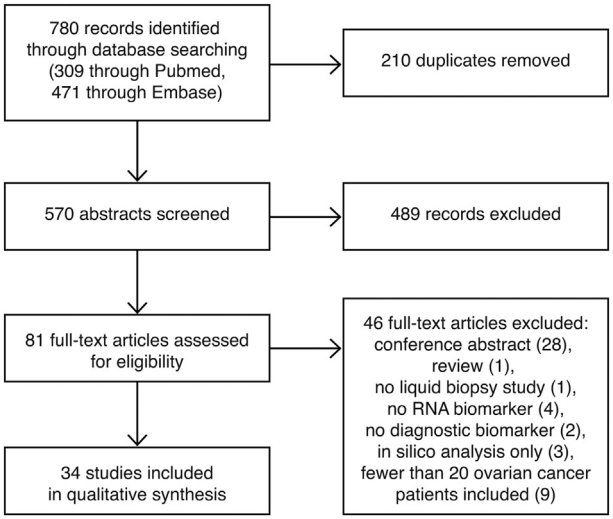

Fig. 2. PRISMA flow diagram.

the early disease setting. In 30 out of the 34 studies, the liquid biopsy was collected in treatment naïve patients and before surgery, reflecting the intended use. In the remaining 6 studies, the timepoint of fluid collection was not mentioned.

3.3. Basic characteristics of the included studies

The vast majority of the studies (34/36) focused on blood-derived fluids: 21 studies included serum samples, two studies focused on extracellular vesicles (EVs) isolated from serum; eight studies included plasma, two studies focused on EVs isolated from plasma and one study focused on both plasma and plasma EVs (Table 1). Only two studies investigated alternative biofluids: one study looked into urine [37] and another one focused on ascites.

Although the extracellular transcriptome of human biofluids is complex and diverse, most liquid biopsy studies in this field focused on miRNAs (30/36). Only two studies looked into circRNAs [23,27], two studies investigated mRNAs [18,38], one study looked into long non-coding RNAs (lncRNAs) [45] and one study looked into a combination of one miRNA, one lncRNA and one mRNA [25].

Surprisingly, RNA sequencing for unbiased biomarker discovery is rarely used in the reported literature on RNA biomarkers for early diagnosis of ovarian cancer. Only three studies used RNA sequencing in combination with RT-qPCR to detect potential biomarkers [14,41,46]. In 25 studies the biomarker detection is based on RT-qPCR only, in five studies both a miRNA microarray and RT-qPCR were used [32,34,35,37,42] and in three studies only a miRNA microarray was used [13,19,25].

Sample sizes of the studies range from 50 (25 ovarian cancer patients versus 25 women with benign ovarian cysts) [20] to 3079 (320 ovarian cancer patients versus 2759 healthy women) [13]. Ideally, if a biomarker test performs well in the discovery cohort, its performance should be evaluated in an independent validation cohort [49]. Only in 9/36 studies the potential biomarker is validated in an independent cohort [13,20,24,26,34,35,37,42,46]. In addition, having results reproduced by an independent research group adds to the robustness of the findings.

3.4. Overlap and discrepancies between the markers reported in different studies

In total, 75 RNA biomarkers have been reported to be differentially abundant in biofluids of ovarian cancer patients compared to liquid biopsy samples of the control group (Table 2). The majority of these RNA biomarkers (39/75) were exclusively higher in ovarian cancer, 26 biomarkers were exclusively lower in ovarian cancer. For 10 biomarkers conflicting results were observed in different studies.

Markers that were reported in at least two studies or two biofluids or that were validated in an independent validation cohort within one study, are considered to be more reliable as candidate marker than markers that are only reported in a single case/control cohort. Biomarkers confirmed to be higher in at least two studies include miR-21, the miR-200 family (miR-200a, miR-200b, miR-200c, miR-141 and miR-429), miR-205, miR-10a and miR-346. Amongst the biomarkers confirmed to be lower in at least two studies are miR-122, miR-193a, miR-223, miR-126 and miR-106b.

The discrepancy observed for 10 biomarkers might be partly due to the difference in included ovarian cancer types, e.g. miR-195, miR-16 and miR-191 are higher in plasma of endometriosis-associated ovarian cancer (either clear cell or endometrioid carcinoma) compared to healthy women [21], while these three markers are lower in plasma or plasma EVs of epithelial ovarian cancer patients (in who pre-existence of endometriosis is not reported) compared to healthy women or women with a benign ovarian mass [16,19,24]. Other potential factors contributing to discrepant results might be the differences in biofluid collection, in methods to identify the RNA molecules and in data-analysis.

3.5. Diagnostic performance of the reported models

Quantifying RNA results in a continuous value, rather than a simple present or absent call. Receiver operating characteristic (ROC) curves are often used to determine the most optimal threshold value as they show the trade-off between sensitivity and specificity for every possible threshold. In case of markers for ovarian cancer, this trade-off refers to wanting to detect as many ovarian cancer cases as possible (true positives) while keeping the number of misclassified non-cancerous cases (false positives) low. Moreover, the area under a ROC curve (AUC) measures the overall performance of a marker, or a combination of markers. A perfect classifier would result in an AUC of 1 while a classifier with an AUC of 0.5 does not perform better than random chance.

Out of the 34 studies in this review, 27 studies performed at least one ROC curve analysis. The AUCs of the best performing classifiers in these studies range from 0.694 to 1 (Suppl. Table 2). Half of the studies report a 95% confidence interval for the AUC value. In order to avoid overfitting, the classifier with its prediction rule should ideally be validated in an independent patient cohort, or in case this cohort is not available, through cross-validation-based methods [49]. However, this is done in less than a third of the studies (five have an independent cohort, four used cross-validation).

Even though CA125 protein is currently the standard diagnostic marker for ovarian cancer, only one third of the studies directly compare the performance of (combinations of) RNA markers to CA125. Individual levels of serum EV miR-145 [15], serum EV miR-200b [15], plasma circBNC2, and serum RPS-8337J1.2 [25] resulted in a higher AUC than that of the CA125 protein. The combination of miR-205 and let-7f was also able to outperform CA125 [42], just like a neural network based on 14 miRNAs [46]. Contrary to the individual RNA markers above, the better performance of these two combined models were each validated in an independent cohort as well. Additionally, several combinations of CA125 with RNA markers are proposed that seem to increase diagnostic performance: serum EV miR-375 + serum EV miR-1307 + CA125 [30]; serum miR-99a-5p + CA125 [47]; plasma KISS1 mRNA + CA125 [18]; plasma miR-200c + plasma miR-221 + CA125 [24]; serum miR-193a-5p + HE4 + CA125 [33]. However, validation in an independent cohort or cross-validation is lacking for all models except the first one.

The best reported individual RNA classifiers are five miRNAs (miR-141, miR-200a, miR-200c, miR-429, and miR-1290) that each have a perfect classifying ability (AUC 1), but this study has no validation cohort and compares RNA in ascites from ovarian cancer patients to RNA

Table 1
Overview of study characteristics and detected higher or lower abundant RNAs in ovarium cancer vs controls.

Reference	Differentially higher abundant	Differentially lower abundant	Biofluid	Technique	FIGO stage (n)			Discovery (n)			Validation (n)		
					I,II	III,IV	UN	OV	HC	BD	OV	HC	BD
Chen et al. [18]	KISS1		plasma	RT-qPCR	12	28		40	20				
El-Shal et al. [25]	RP5-837 J1.2	miR-361-3p, PELI3	serum	RT-qPCR	24	26		50	45	42			
Elias et al. [46]			serum	RT-qPCR, microarray, RNA seq	142[a]	149[a]		255	138	111[b]	25	0	26
Fan et al. [27]	circMAN1A2		serum	RT-qPCR			36	36	36				
Galdiero et al. [38]	HMGA2		plasma	RT-qPCR	11	36		47	23				
Gao and Wu et al. [40]	miR-141, miR-200c		serum	RT-qPCR	54	20		74	50	19[b]			
Guo et al. [43]	miR-92		serum	RT-qPCR	35	15		50	50				
Hu et al. [23]		circBNC2	plasma	RT-qPCR	25	58		83	83	83			
Ji et al. [41]	miR-22, miR-93	miR-106b, miR-451	serum	RNA seq, RT-qPCR	7	24		31	8	23			
Kan et al. [44]	miR-200a, miR-200b, miR-200c		serum	RT-qPCR	1	27		28	28				
Kim et al. [15]	miR-93, miR-145, miR-200c		serum EV	RT-qPCR	17	31		48	20				
Kobayashi et al. [31]	miR-1290		serum	RT-qPCR	37	33		70	13				
Langhe et al. [20]		let-7i-5p, miR-25-3p, miR-122, miR-152-5p	serum	RT-qPCR	6	19		5	5		20	20	
Liang et al. [39]		miR-145	serum	RT-qPCR	31	53		84	135	51			
Liu et al. [45]	LOXL1-AS1		plasma	RT-qPCR	118	67		185	43				
Mahmoud et al. [17]	miR-21		serum	RT-qPCR	21[a]	40[a]		60	30				
Márton et al. [28]	miR-34a, miR-34b, miR-141, mir-200a, miR-200b, miR-200c, miR-429, miR-203a		plasma	RT-qPCR			28	28	60	12			
Meng et al. [36]	miR-7, miR-429	miR-25, miR-93	serum	RT-qPCR	32	147	1	180	66				
Oliveira et al. [24]	miR-21-5p, miR-200c-3p, miR-221-3p, miR-484	miR-195-5p, miR-451a	plasma	RT-qPCR	13	177		95	95		95	95	
Paliwal et al. [12]	miR-21	miR-22	serum	RT-qPCR	32	48		80	80				
Pan et al. [16]	miR-21, miR-100, miR200b, miR-320	miR-16, miR-93, miR-126, miR-223	plasma EV	microarray	72	20	13	106	29				
Ren et al. [33]		miR-193a-5p	serum	RT-qPCR	12[a]	26[a]	27[a]	45	40	30			
Shapira et al. [19]	miR-1274a, miR-625-3p, miR-720	miR-16, miR-17, miR-19b, miR-20a, miR-24, miR30b, miR-30a-5p, miR-30c, miR-92a, miR-106a, miR-106b, miR-126, miR-146a, miR-150, miR-191, miR-193a-5p, miR-223, miR-320, miR-328	plasma	microarray	6	36		42	23	36			
Su et al. [30]	miR-375, miR-1307		serum EV	RT-qPCR	8	42		50	50	50			
Suryawanshi et al. [21]	miR15b, miR-16, miR-21, miR-191, miR-195, miR-1973, miR-1974, miR-1977, miR-1979, miR-4284		plasma	RT-qPCR	11	24		35	20	33			
Todeschini et al. [34]	miR-595, miR-1246, miR-2278		serum	microarray, RT-qPCR		168		110	52		58	13	
Wang et al. [26]	miR-10a-5p, miR-145-5p, miR-205-5p, miR-328-3p, miR-346		plasma, plasma EV	RT-qPCR	34	77		32	34		69	66	
Xu et al. [22]	miR-21		serum	RT-qPCR	32	62		94	40				
Yokoi et al. [13]			serum	microarray	115	218		160	1379		173	1380	95
Yoshimura et al. [47]	miR-99a-5p		serum	RT-qPCR	31	31		61	20	26			
Záveský et al. [32]	miR-30a-5p, miR-200a, miR-200b, miR-200c, miR-141, miR-429, miR-1290		ascites[c]	microarray, RT-qPCR	6	17		23	34				
Zhang et al.	let-7d-5p, miR-93-5p,	miR-99b-5p, miR-122-5p, miR-185-5p	plasma	RNA seq,	6	24		30	30				

(continued on next page)

Table 1 (*continued*)

Reference	Differentially higher abundant	Differentially lower abundant	Biofluid	Technique	FIGO stage (n)			Discovery (n)			Validation (n)		
					I,II	III,IV	UN	OV	HC	BD	OV	HC	BD
[14]	miR-106a-5p		EV	RT-qPCR									
Zheng et al. [42]	miR-205	let-7f	serum	microarray, RT-qPCR	133	227		76	30		284	170	
Zhou et al. [37]	miR-30a-5p	miR-6076	urine	microarray, RT-qPCR	16	18	5	5	5		34	25	26
Zhu et al. [35]	miRNA-125b		serum	microarray, RT-qPCR	33	102		18	16		135[d]	54[d]	
Zuberi et al. [29]		miR-145	serum	RT-qPCR	33	37		70	70				

EV, extracellular vesicles; FIGO, International Federation of Gynecology and Obstetrics; UN, unknown; HC, healthy control; BD, benign gynecological disease; OV, ovarian cancer.
[a] Sum of reported subtypes is higher than reported number of ovarian cancer patients in study.
[b] Including borderline ovarian cancer.
[c] Ascites in ovarium cancer patients, plasma in healthy controls.
[d] Not completely independent - samples of discovery cohort also included in validation.

Table 2
Overview of reported RNA biomarkers in biofluids for early diagnosis of ovarian cancer.

RNA markerRNA marker	Biofluid		RNA markerRNRNA markerA	Biofluid	
miR-21	plasma [21,25], plasma EV [16], serum [12,17,22]	++	miR-122	plasma EV [14], serum [20]	--
miR-200a	ascites [32], plasma [28], serum [44]	++	miR-193a	plasma [19], serum [33]	--
miR-200b	ascites [32], plasma [28], serum [44]	++	miR-223	plasma EV [16], plasma [19]	--
miR-200c	ascites [32], plasma [24,28], serum [40,44], serum EV [15]	++	miR-126	plasma EV [16], plasma [19]	--
			miR-106b	plasma [19], serum [41]	--
miR-141	ascites [32], plasma [28], serum [40]	++	let-7f	serum [42]	--
miR-429	ascites [32], plasma [28], serum [36]	++	let-7i	serum [20]	--
miR-205	plasma [26], plasma EV [26], serum [42]	++	miR-6076	urine [37]	--
miR-10a	plasma [26], plasma EV [26]	++	miR-451a	plasma [24]	--
miR-346	plasma [26], plasma EV [26]	++	miR-152	serum [20]	--
miR-221	plasma [24]	++	miR-25	serum [20,36]	--
miR-484	plasma [24]	++	miR-185	plasma EV [14]	-
miR-1246	serum [34]	++	circBNC2	plasma [23]	-
miR-595	serum [34]	++	miR-99b	plasma EV [14]	-
miR-2278	serum [34]	++	miR-451	serum [41]	-
miR-125b	serum [35]	++	miR-361	serum [25]	-
circMAN1A2	serum [27]	+	PELI3	serum [25]	-
HMGA2	plasma [38]	+	miR-146a	plasma [19]	-
let-7d	plasma EV [14]	+	miR-150	plasma [19]	-
miR-15b	plasma [21]	+	miR-17	plasma [19]	-
miR-1977	plasma [21]	+	miR-19b	plasma [19]	-
miR-1979	plasma [21]	+	miR-20a	plasma [19]	-
miR-1973	plasma [21]	+	miR-24	plasma [19]	-
miR-1974	plasma [21]	+	miR-92a	plasma [19]	-
miR-4284	plasma [21]	+	miR-30b	plasma [19]	-
KISS1	plasma [18]	+	miR-30c	plasma [19]	-
miR-1307	serum EV [30]	+			
miR-375	serum EV [30]	+			
miR-34b	plasma [28]	+	miR-93	plasma EV [14], serum EV [15], serum [41]	++
miR-34a	plasma [28]	+		plasma EV [16], serum [36]	--
miR-203a	plasma [28]	+	miR-145	plasma [26], plasma EV [26], serum EV [15]	++
miR-1290	ascites [32]	+		serum [29,39]	--
miR-100	plasma EV [16]	+	miR-30a	ascites [32], urine [37]	++
miR-7	serum [36]	+		plasma [19]	--
RP5-837 J1.2	serum [25]	+	miR-16	plasma [21]	+
miR-1274a	plasma [19]	+		plasma EV [16], plasma [19]	--
miR-625	plasma [19]	+	miR-195	plasma [21]	+
miR-720	plasma [19]	+		plasma [24]	--
LOXL1-AS1	plasma [46]	+	miR-328	plasma [26], plasma EV [26]	++
miR-99a	serum [47]	+		plasma [19]	-
			miR-106a	plasma EV [14]	+
				plasma [19]	-
			miR-22	serum [41]	+
				serum [12]	-
			miR-320	plasma EV [16]	+
				plasma [19]	-
			miR-191	plasma [21]	+
				plasma [19]	-

For each RNA molecule the biofluid in which it was studied and the study reference is shown. The + and - signs correspond to higher and lower abundance, respectively, in ovarian cancer samples versus controls. Markers that were consistently higher or lower in at least two studies, or in at least two biofluids, or that were validated in an independent cohort within one study, are considered more reliable and are highlighted with ++ or –.

in plasma from healthy controls [32]. The specific setup of the study may have resulted in a biofluid classifier (ascites versus plasma) rather than a classifier for ovarian cancer. The diagnostic performance of two of these miRNAs, miR-200c and miR-1290, was also assessed in serum samples and resulted in an AUC of 0.8 [5,40] and even below 0.5 (worse than random chance) [31], respectively.

Differential RNA abundance on its own does not guarantee good performance as classifier. However, combining several of these RNAs in a model seems to improve performance in most studies. The best performing model with independent validation is a model that contains 10 miRNAs in serum (miR-320a, miR-665, miR-3184-5p, miR-6717-5p, miR-4459, miR-6076, miR-3195, miR-1275, miR-3185, and miR-4640-5p) [13]. This model has a perfect AUC of 1 in both training and validation set (sensitivity 0.99, specificity 1). The second-best performing model with validation (leave-one-out cross-validation) combines four miRNAs in serum: miR-7, miR-429, miR-25, miR-93 (AUC 0.98, sensitivity 0.93, specificity 0.92) [36].

3.6. Involvement of the reported biomarkers in ovarian cancer pathogenesis

A perfect understanding of the function of a biomarker is not an absolute requirement for its routine clinical use but it may help researchers to understand the mechanisms of pathogenesis and progression of ovarian cancer. Non-coding RNAs regulate gene expression and have been linked to known oncogenic pathways in various cancer types, including ovarian cancer [50,51]. RNA markers that are involved in one or more hallmarks of cancer, as defined in the foundational work of Hanahan and Weinberg [52], are shown in Fig. 3. Here, we focus on the role of these RNA molecules in ovarian cancer. Pinpointing the exact function of one specific miRNA is not straightforward, as one miRNA can affect, by direct or indirect effects, the expression of a great number of genes. Fig. 3 only reflects a selection of miRNAs and a non-exhaustive view on their putative role(s) in ovarian cancer.

In ovarian cancer, miR-30a has been assigned a pro-oncogenic function by sustaining proliferative signaling through targeting FOXL2 and FOXD1 [53,54]. This miRNA was observed to be higher in urine samples, ascites samples, tissue samples and cell lines of ovarian cancer, while it is lower in plasma of endometriosis-associated cancer patients [21,32,37,53,54]. In other cancer types, including breast cancer, small cell lung cancer and colorectal cancer, miR-30a functions as a tumor suppressor [53].

MiR-221 has been associated with cell proliferation and inhibition of apoptosis of cancer cells by targeting B-cell lymphoma 2 modifying factor (BMF) [55]. MiR-221 is higher in plasma of ovarian cancer patients [16].

The miR-200 family consists of 5 members (miR-200a, miR-200b, miR-200c, miR-141 and miR-429), all linked to several cancer hallmarks. All 5 miRNAs are higher in ascites, plasma and serum [24,28,32,40,44]. MiR-141 and miR-200a have recently been associated with ovarian tumorigenesis by controlling the oxidative stress response through their target p38α [56]. MiR-200a might exert its pro-oncogenic function through modulating PTEN, PCDH9 and ING5, resulting in cancer cell invasion and angiogenesis [57–59]. The miR-200 family, especially miR-200c and miR-429, potentially regulates epithelial-to-mesenchymal transition by targeting the E-cadherin repressor ZEB2 [60].

Conflicting differential abundance patterns of miR-328 have been reported in plasma samples of ovarian cancer patients [19,26]. In cancer stem cells, miR-328 exerts a pro-oncogenic function through the inhibition of DNA damage binding protein 2 (DDB2)[61].

MiR-145 has been reported to be higher in plasma, plasma EVs and serum EVs of ovarian cancer patients in two studies [15,26], while it was lower in serum of ovarian cancer patients in two other studies [29,39]. In ovarian cancer tissue, miR-145 was downregulated

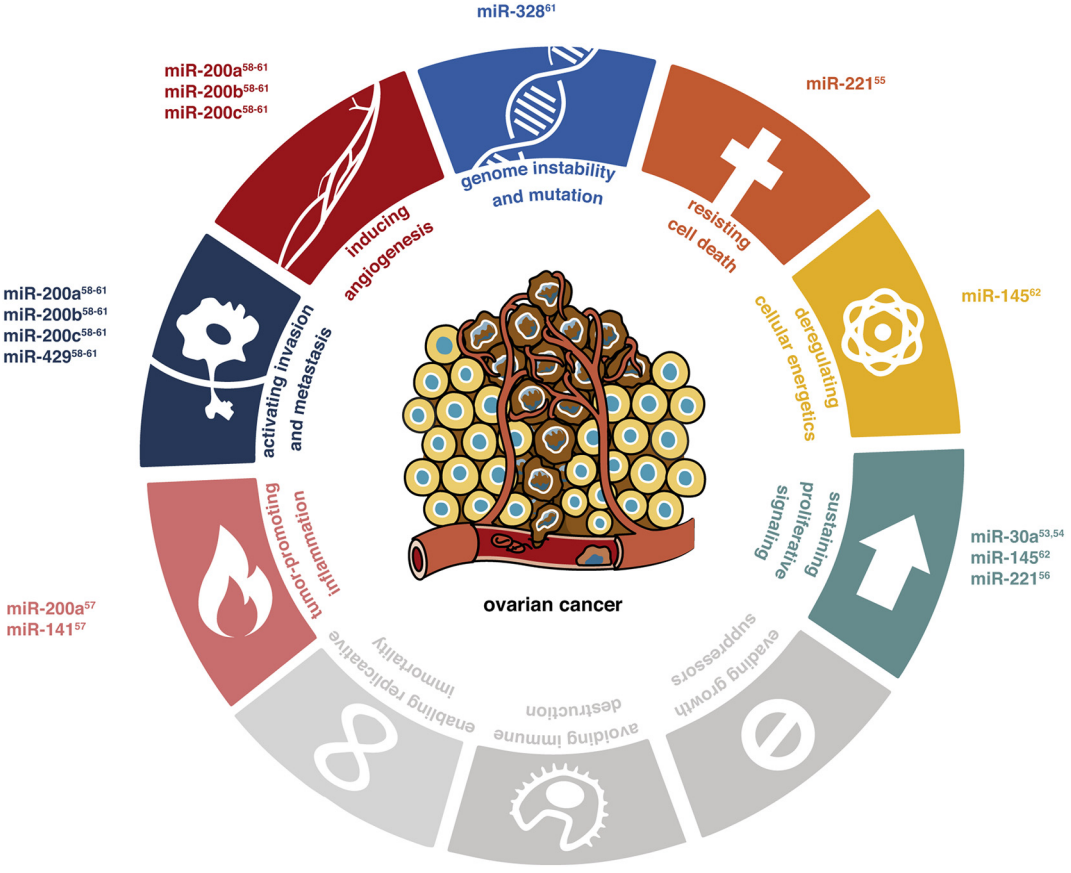

Fig. 3. RNA markers affect several hallmarks of cancer, reported by Hanahan and Weinberg [52], and contribute to the pathogenesis of ovarian cancer.

compared to normal ovarian tissue. MiR-145 has a tumor suppressor function by affecting the cancer cell metabolism. Overexpression of miR-145 in ovarian cancer cell lines inhibits glutamine consumption and cellular ATP levels through targeting MYC [62].

4. Discussion

This review summarizes the literature regarding RNA biomarkers detectable in human biofluids for early diagnosis of ovarian cancer. A limitation of this review is the heterogeneity of the included studies in terms of methodology used. Robustness of procedures used for sample processing and data analysis strongly influences the reproducibility of biomarker studies [49]. Pre-analytical variables, such as blood collection tube, volume, centrifugation speed and duration, are known confounders and should therefore be clearly reported [63]. Moreover, discrepancies in protocols between samples from cases and controls or between samples collected in multiple centers can bias the final results and hamper direct comparison with other studies [64]. We assessed the level of reproducibility for the liquid biopsy collection, RNA isolation procedure and data-analysis in all studies (supplementary Table S2). Remarkably, the liquid biopsy collection, RNA isolation procedure and data-analyses can only be fully reproduced in one study based on the descriptions in the manuscript [42]. In 33/36 studies, at least one methodological aspect of the biofluid collection (collection tube, volume, centrifugation speed, centrifugation duration) is missing. In 21/36 studies, the input volume of the RNA isolation and/or the RNA isolation kit are not mentioned. General data analysis procedures are reported in the majority of studies (30/36) although some subparts of it, for example the presence or absence of multiple testing correction, are not always clearly mentioned. In the absence of gold-standard protocols, we recommend disclosing detailed information of the sample collection methods, RNA isolation and profiling methods as well as the data analyses. This will facilitate future identification, evaluation and development of liquid biomarkers.

Screening of an asymptomatic average- or high-risk population for early detection of cancer should be clearly distinguished from diagnostic work-up of patients with known pelvic mass. For the latter, the advantages of liquid biopsies over standard surgical biopsies cannot be underestimated, as they are non-invasive, simple to perform, more patient-friendly and allow for serial testing. Thirty-four of the included liquid biopsy studies in this review focus on blood-derived fluids. While urine and ascites were investigated in two studies [32,37], the biomarker potential of these alternative biofluids for early detection of ovarian cancer is still mainly unexplored. Ascites is typically seen in advanced disease, and it is therefore not suitable for early detection of cancer, though it may highlight biomarkers that need to be validated in other liquid biopsies. Utero-tubal lavage fluid on the other hand, might be an interesting biofluid to further investigate and profile because it shows proximity to the tumor and its protein content has biomarker potential [65]. The low frequency of early stage high grade serous ovarian cancer, which is an inevitable result of the lack of screening, is also the most challenging limitation of this research field. To date there are no reports of large cohorts of liquid biopsies from early-stage cases, or of samples taken shortly prior to ovarian cancer diagnosis, though large efforts are ongoing (e.g. the STRIVE trial, NCT03085888 [66]). These biofluid collections are key to advance the development of a clinically meaningful screening tool, which will actually reduce ovarian cancer related mortality. As the included studies combine samples obtained from patients with early disease and advanced disease, the potential biomarkers identified so far, should still be validated in a cohort consisting only of early-stage ovarian cancer samples. Biomarkers identified in patients with advanced disease are not necessarily relevant for the early disease setting. The diagnostic performance of LOXL1-AS1, a potential biomarker identified in a cohort of 119 early-stage patients, 67 advanced patients and 43 healthy women, is clearly higher in patients with advanced disease compared to patients with early-stage disease [45]. However, we cannot exclude that some current or future biomarkers may exist that are useful for identification of both early and advanced disease, albeit with different sensitivity. For example, KISS1 mRNA is identified as potential biomarker in a cohort of 40 ovarian cancer patients (12 early-stage patients and 28 advanced-stage patients) and 20 healthy women. The diagnostic performance of KISS1 mRNA is higher in the early-stage group (AUC 0.879) compared to the advanced-stage group (AUC 0.728) [18]. Confirmation of these findings in an independent validation cohort of patients with early disease is needed. The majority of the reported RNA markers are miRNAs, referred to in papers only by their name. However, unambiguous reporting of a miRNA can only be achieved by either disclosing the sequence or, alternatively, providing its name or accession number explicitly in combination with the release version of the miRBase database as these may change over time [67]. Annotation of mRNAs, lncRNAs and circRNAs might also change in the future so attention for unambiguous reporting of these RNA biotypes is important as well.

To date, studies looking into the biomarker potential of circulating RNAs for early detection of ovarian cancer are exploratory in nature and identify altered levels of circulating RNAs in ovarian cancer patients versus a control group. Although 75 RNA biomarkers have been identified as candidate diagnostic biomarkers in literature and part of these RNA biomarkers have been confirmed in independent studies, additional validation steps are needed for all markers before application in clinical setting is possible. These liquid biopsy tests have not yet demonstrated analytical validity, clinical validity and clinical utility [49]. Analytical validation describes how accurately and reliably the test measures the marker(s) of interest in the patient sample. Clinical validation assesses whether the biomarker can distinguish ovarian cancer patients from all other individuals without ovarian cancer. Clinical utility describes the likelihood that a biomarker test will enable clinicians to make decisions that ultimately reduce morbidity and mortality. Early detection of cancer is widely believed to result in better patient outcomes, but this remains to be demonstrated in each particular case. Identifying candidate biomarkers is a first important step that has already been initiated in the ovarian cancer field. Making sure biomarker tests also impact clinical management, and ideally patient quality of life or overall survival, still is a hurdle to take [49].

With only three studies using RNA sequencing to profile the extracellular transcriptome in serum and plasma EVs of ovarian cancer patients [14,41,46], we believe RNA sequencing of biofluids of ovarian cancer patients is still an untapped resource for biomarker discovery. Instead of focusing on a single RNA marker like most of the included studies, the answer may lie in a combination of multiple biomarkers or even multi-omics biomarker signatures.

We believe that further characterizing the circulating transcriptome of biofluid samples from ovarian cancer patients is mandatory to allow discovery and implementation of clinically useful biomarkers in this field. In addition, the identified markers may trigger further research elucidating the pathological processes and potential preventive or therapeutic interventions. Prospective studies with standardized procedures and large sample cohorts are warranted to enhance the consideration of the clinical significance of circulating RNAs in ovarian cancer.

5. Conclusions

Seventy-five RNA markers have been reported in human biofluids as potential biomarkers for early diagnosis of ovarian cancer. Candidate RNA biomarkers that were higher in biofluids of ovarian cancer patients compared to control samples in at least two independent studies include miR-21, the miR-200 family, miR-205, miR-10a and miR-346. Amongst the markers that are lower in at least two studies are miR-122, miR-193a, miR-223, miR-126 and miR-106b. While some RNA-based signatures have an improved diagnostic performance compared

to CA125, further validation of the reported markers is required before implementation in routine clinical care.

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Author contribution

Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualisation, Project administration: E.H. and A.M.; Funding acquisition, Supervision: J.V., P.M., K.L.; Writing - original draft: E.H. and A.M.; Writing - review & editing: J.V., P.M., K.L.Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Visualisation: E.H. and A.M.; Funding acquisition, Supervision: J.V., P.M., K.L.; Writing - original draft: E.H. and A.M.; Writing - review & editing: J.V., P.M., K.L.

Declaration of Competing Interest

The authors have no competing interests to declare.

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